**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of

DENNY et al

Atty. Ref.: 5011-8; Confirmation No. 1493

Appl. No. 10/529,772

TC/A.U. 1626

Filed: June 2, 2005

Examiner: Kosack, J.R.

For: NITROANILINE-BASED ALKYLATING AGENTS AND THEIR USE AS PRODRUGS

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

**DECLARATION**

I, William R. Wilson, hereby declare and state that:

1. I am a co-applicant in the above application.

2. The following experiments have been performed under my direct supervision and control.

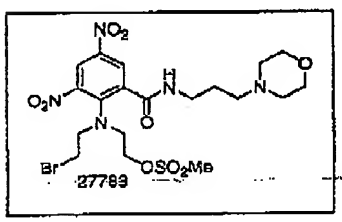
3. **Cell line growth inhibition ($IC_{50}$) Assays.** All *in vitro* studies used aliquots of frozen solutions of compounds in DMSO, with a maximum DMSO concentration in culture of ≤ 0.5%. Compounds were evaluated for cytotoxicity (measured as $IC_{50}$ values following an 18 h drug exposure) in the mammalian (Chinese hamster fibroblast) cell line T79-A3, stably transfected with the E. coli *nfsB* nitroreductase (NTR), and the corresponding wild-type line T78-1 (transfected with an empty shuttle vector). $IC_{50}$ assays were performed by seeding log-phase cells in 96-well plates in 50 µl αMEM containing 5% fetal bovine serum (EBS; 50-800

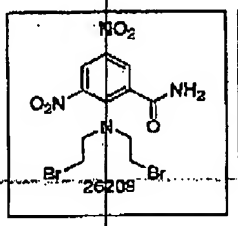
cells/well). After growth for 18 hr, prodrugs were added from DMSO stock solutions which were diluted into culture medium immediately prior to addition to the cell cultures. Drug exposure was terminated by washing with fresh medium after 18 hr, and cultures were grown for a further 3 days before staining with sulforhodamine B to assess cell density. The $IC_{50}$ was determined as the interpolated drug concentration required to reduce absorbance to 50% of that of controls on the same plate. Selective cytotoxicity for the $NTR^{+ve}$ cell line was assessed from the intra-experiment ratio of $NTR^{-ve}/NTR^{+ve}$ $IC_{50}$s for the cell line pair.

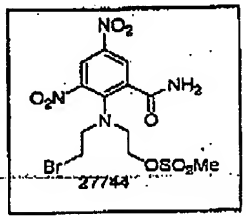
Table 1. $IC_{50}$ values (μM) and $IC_{50}$ ratios

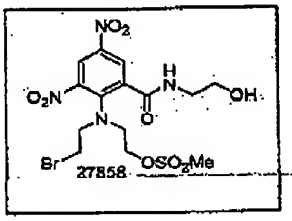
| Compd | T78-1 (μM) | n | T79-A3 (μM) | n | ratio |
|---|---|---|---|---|---|
| | | | | | |
| 26209 | 17.5* | 4 | 0.19* | 4 | 92* |
| 27744 | 24.2±2.2 | 3 | 0.0048±0.0014 | 3 | 5000 |
| 27858 | 63.7±6.2 | 3 | 0.0285±0.0045 | 2 | 2235 |
| 27783 | 52.2±5.9 | 3 | 0.285±0.132 | 2 | 183 |
| 27555 | 169±9 | 3 | 0.096±0.047 | 3 | 1760 |

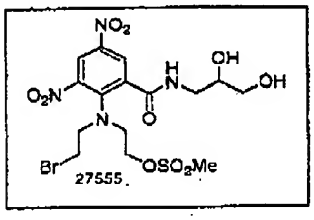
*data from Friedlos et al., for a 24h drug exposure



4. The results show that the bromomesylate 27744, directly comparable to the dibromo compound 26209 (compound 13 in Friedlos *et al.*) is roughly equipotent in the wild-type cell line, but dramatically more effective (39-fold) in the NTR-transfected line. The similar bromomesylate 27858, while less toxic in the wild-type line, is again much more potent than the dibromo in the NTR-transfected line. The morpholide 27783 was much less toxic, but still showed high differential activity, whereas the diol 27555 was less toxic in the wild-type cell line but more potent than the dibromo in the NTR-transfected line.

I declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

William R. Wilson

9 DEC 2008
Date